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Short communication

Satellite DNA-based species-specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae)

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Abstract

The pinewood nematode *Bursaphelenchus xylophilus* is a severe pest of coniferous trees, and has been designated as a quarantine organism in the European Union. From the sequence of a satellite DNA family characterized in the genome of this nematode, we developed a PCR procedure that allowed the specific discrimination of this species from closely related *Bursaphelenchus* species found on coniferous trees. Moreover, because of the repetitive nature of satellite DNA, positive amplification was achieved from *B. xylophilus* single individuals, which should contribute to an easy diagnostic procedure for assisting in the management of this major pest of conifer forests.

The nematode *Bursaphelenchus xylophilus* is the causal agent of pine wilt disease, one of the major conifer diseases worldwide. This nematode is a quarantine organism in the European Union. So far, it has been reported only in one restricted area in Portugal in 1999 (Mota et al., 1999). Among the 25 *Bursaphelenchus* species found in coniferous trees in Europe, *B. mucronatus* is the most abundant in central and eastern Europe, while *B. sexdantati* and *B. leoni* occurred most frequently in Southern Europe (Mota et al., 1999). Because of their morphological resemblance, the taxonomic status of *Bursaphelenchus* species remains unclear. For example, *B. mucronatus* has frequently been confused with *B. xylophilus*, the only difference being their distinctive tail terminus (Mamiya and Enda, 1979). Moreover, due to incomplete reproductive isolation, these species have been proposed as a supraspecies referred to as the pine wood nematode species complex (De Guiran and Bruguier, 1989). In order to provide a more comprehensive view of the relationships between the pathogenic and non-pathogenic species within the genus, it is necessary to first distinguish *B. xylophilus* from the other *Bursaphelenchus* species. In the present study, a species-specific set of primers was developed to amplify a repetitive DNA family in the genome of *B. xylophilus* that unambiguously discriminates single individuals of this nematode from the closely related *Bursaphelenchus* species also found on coniferous trees.

Genomic DNA was extracted from pooled nematodes of each isolate tested, i.e. three isolates of *B. xylophilus*, and one isolate of *B. mucronatus*, *B. leoni* and *B. tusciae*, respectively, using the phenol/chloroform method (Sambrook et al., 1989). PCR primers were designed close to both ends of the sequence of the 160-bp monomer of the satellite DNA family previously characterized in *B. xylophilus* (Tarès et al., 1993; GenBank accession L09652); J10-1, 5′-GGTGTCTAGTATAA TATCAGAG-3′ and J10-2Rc, 5′-GTGAAT TAGTGACGACGGAGTG-3′. PCR was carried out in 25 μl reaction mixtures containing 10 ng of template DNA, 50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 200 mM dNTP, 250 ng of each of the primers and 1 Unit of *Taq* DNA polymerase (Q-Biogene) using a TRIO-Thermoblock

thermal cycler (Biometra). After denaturation at 94 °C for 5 min, cycling was performed for 25 cycles of 30 s at 94 °C, 1 min at 64 °C and 1 min at 72 °C, with a postcycling extension at 72 °C for 5 min. Amplification was also performed on individual nematodes, prepared according to a single worm PCR procedure modified from Williams et al. (1992). Briefly, single nematodes were transfered to a dry thin walled PCR tube, covered with 2.5 μl lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 60 mg ml^{-1} proteinase K, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin) and overlaid with mineral oil. Tubes were put at −80 °C for 45 min, and immediately transfered to 60 °C for 60 min and then 95 °C for 15 min in the thermal cycler. In order to show that template of an integrity that can support PCR amplification was obtained using the single worm procedure, one half of the template prepared from each sample was amplified with the satellite DNA primers and conditions described above, and the other half was amplified with standard rDNA ITS primers and conditions (Mota et al., 1999).

Since the satellite DNA family had been shown to be constituted of repeats organized in tandem arrays (Tarès et al., 1993), the amplification of a ladder of multimers of the 160-bp monomer was expected in a PCR experiment using the selected primers. Indeed, with primers J10-1 and J10-2Rc, ladder patterns of monomers and multimers of the expected size were amplified from genomic DNA of the *B. xylophilus* isolates only, while no amplification was detected with the other *Bursaphelenchus* species tested (data not shown). To test the sensitivity of this procedure, amplification of DNA from single nematodes was considered. Use of proteinase K, in combination with the alternation of high and low temperatures, proved to be efficient to make the genomic DNA of a single individual suitable as a template for PCR. PCR products from *B. xylophilus* single nematodes obtained this way were thus compared to those obtained from *B. xylophilus* phenol/chloroform purified genomic DNA. As shown in Figure 1, individual amplification patterns were identical to the one obtained from *B. xylophilus* genomic DNA, although some differences in banding intensity could occur. To confirm that amplification using J10-1 and J10-2Rc as primers was *B. xylophilus* -specific, PCR was carried with single nematodes from different *Bursaphelenchus* species and isolates. In particular, one *B. xylophilus* isolate from Japan (J10) and two from Canada (01-667-1 and 01-602-1) were used, respectively. As expected, amplification was detected only in lanes corresponding to the three *B. xylophilus* isolates, and a regular ladder pattern was obtained. In contrast, no amplification occurred in samples belonging to other *Bursaphelenchus* species prepared in the same manner (Figure 1). Moreover, amplification

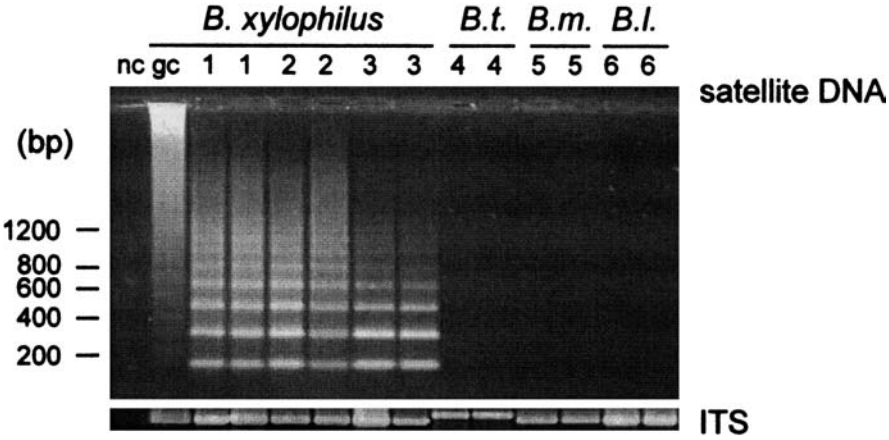

Figure 1. PCR amplification from single nematodes using the satellite DNA-based primer set specific for *Bursaphelenchus xylophilus*. nc, negative control; gc, genomic DNA purified from pooled *B. xylophilus* individuals. Lanes 1, *B. xylophilus* (isolate J10); lanes 2, B. xylophilus (isolate 01-667-1); lanes 3, *B. xylophilus* (isolate 01-602-1); lanes 4, *B. tusciae*; lanes 5, *B. mucronatus*; lanes 6, *B. leoni*. Top: For *B. xylophilus* samples only, a typical ladder pattern was amplified, that resulted from the tandem organization of the 160-bp repeats. Bottom: for all samples, positive amplification using rDNA ITS primers.

of rDNA occurred in all samples, which indicated that samples from *B. leoni*, *B. mucronatus* and *B. tusciae* were indeed true negatives, and that the lack of amplification with satellite DNA primers in these reactions did not result from either failed DNA extractions or PCR inhibition (Figure 1).

The results obtained in the present study illustrate the specific distribution of satellite DNA sequences already documented between closely related taxa of nematodes of agronomic interest (Grenier et al., 1997). Moreover, because of the repetitive nature of this satellite DNA family (up to 30% of the nematode genome; Tarès et al., 1993), positive amplification was achieved from single nematodes. The primer set developed here will be useful in positively identifying *B. xylophilus* in samples collected in the wild, and could contribute to the development of a simple diagnostic procedure for this quarantine pest. In addition, this result represents a crucial step to provide a more comprehensive understanding of the origin and evolution of the pine wood nematode species complex in Europe.

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